INFECTION AND IMMUNITY, Feb. 2006, p. 1009–1015 Vol. 74, No. 2
0019-9567/06/$08.00+0 doi:10.1128/IAI.74.2.1009–1015.2006

Cytokine Response and Survival of Mice Immunized with an Adenovirus Expressing *Bacillus anthracis* Protective Antigen Domain 4

Michael J. McConnell,[1,2] Philip C. Hanna,[2] and Michael J. Imperiale[1,2]*

Department of Microbiology and Immunology[2] and Comprehensive Cancer Center,[1] University of Michigan Medical School, Ann Arbor, Michigan 48109-0942

Received 26 September 2005/Returned for modification 29 October 2005/Accepted 5 November 2005

Adenovirus vectors are promising for use in vaccinating against potential agents of bioterrorism and emerging infections because of their proven safety in humans and their ability to elicit rapid immune responses. Here, we describe the construction and evaluation of an adenovirus vaccine expressing domain 4 of *Bacillus anthracis* protective antigen, Ad.D4. Ad.D4 elicited antibodies to protective antigen 14 days after a single intramuscular injection, which were further increased upon boosting. Furthermore, two doses of Ad.D4 4 weeks apart were sufficient to protect 67% of mice from toxin challenge. Additionally, we have characterized the release of inflammatory cytokines from vaccinated mice after lethal-toxin challenge. We demonstrate that interleukin 1β (IL-1β) levels in mice that survive lethal toxin challenge are similar to levels in nonsurvivors and that IL-6 levels are higher in survivors than in nonsurvivors. These findings suggest that lethal-toxin-mediated death may not be a direct result of inflammatory-cytokine release.

Inhalational anthrax, a disease caused by the spore-forming bacterium *Bacillus anthracis*, is highly lethal and has emerged as a serious bioterrorism threat, as evidenced by the intentional dispersal of weaponized spores through the U.S. mail system in 2001. After the onset of detectable symptoms, the clinical course of inhalational anthrax is extremely rapid, usually resulting in sudden death within a few days (6). This rapid disease progression severely limits the effectiveness of postexposure antimicrobial therapies. A Food and Drug Administration-licensed vaccine for anthrax, the Anthrax Vaccine Adsorbed (AVA), is available in the United States and consists of the filtered culture supernatant from a nonencapsulated strain of *B. anthracis* combined with an aluminum adjuvant (21). AVA is effective in protecting animals against aerosolized spores, but a number of drawbacks associated with its use have prompted the Institute of Medicine to call for the development of a new vaccine formulation (22). First, AVA is standardized by a potency assay in which its ability to provide protection against spore challenge is assessed (9). The components present in each production batch of AVA are thus poorly characterized. Second, AVA is associated with a number of complications upon subcutaneous administration, often resulting in painful reactions at the site of injection, in addition to systemic reactions, such as joint pain and intestinal disorders (12, 33, 42). These side effects are thought to be due to bacterial components present in the vaccine, as well as the use of an aluminum adjuvant. Finally, AVA requires a complicated and protracted dosing schedule, which consists of six injections over 18 months plus yearly boosts, although it has been reported that two injections are sufficient for stimulating a humoral response (34). A vaccine with fewer side effects that can induce protective immunity using a shorter dosing schedule would be better suited for mass vaccination prior to, or in response to, a bioterrorism event.

After entering the lung, anthrax spores are engulfed by alveolar macrophages, in which the spores germinate into the vegetative form. Upon gaining access to the blood, the bacilli secrete the three subunits that make up the anthrax toxins, protective antigen (PA), edema factor (EF), and lethal factor (LF) (6). The toxins are AB-type toxins in which the common subunit, PA, oligomerizes and subsequently associates with EF or LF to form edema toxin and lethal toxin (LeTx), respectively. It has been well established that antibodies generated against PA are sufficient for providing protection against toxin and spore challenge in animal models of anthrax (32, 38, 48). The crystal structure of PA has been solved, revealing four structural domains within the molecule (29). Furthermore, the structure of PA in complex with its cellular receptor, CMG2, indicates that domain 4 (D4) is primarily responsible for mediating contact between PA and its receptor (40), and deletion of D4 from the *B. anthracis* genome results in a >10,000-fold decrease in virulence (3). Immunization with D4 alone is sufficient to protect mice from spore challenge (10), suggesting that D4 contains the epitopes necessary for generating protective immunity to *B. anthracis*.

Mutation of the LF gene results in a decrease in virulence of over 3 log units, suggesting that LeTx is the bacterial product responsible for much of the morbidity of anthrax (30). Additionally, administration of purified LeTx reproduces many of the disease symptoms associated with anthrax, including rapid death (24). While it is known that LF is a zinc metalloprotease that inactivates MAPK kinases (7), the mechanism by which LeTx ultimately kills an infected host is unclear. Macrophages of some strains of inbred mice are exquisitely sensitive to LeTx-mediated lysis, while the macrophages of other strains

* Corresponding author. Mailing address: University of Michigan Medical School, 6304 Cancer Center, 1500 E. Medical Center Dr., Ann Arbor, MI 48109-0942. Phone: (734) 763-9162. Fax: (734) 615-6560. E-mail: imperial@umich.edu.

are more resistant (11). Furthermore, depletion of macrophages from susceptible mice results in their resistance to LeTx (15). These findings, in addition to work indicating that macrophages release proinflammatory cytokines in response to LeTx, have suggested a model in which LeTx induces a shock-like syndrome due to cytokine release caused by macrophage lysis (15). However, more recent reports have shown that LeTx does not stimulate cytokine release from macrophages but actually inhibits cytokine release caused by lipopolysaccharide (8, 27). Additionally, Moayeri and colleagues showed that LeTx causes death by inducing hypoxic liver failure in strains harboring either toxin-sensitive or -resistant macrophages. This study also showed that death from LeTx was independent of the release of inflammatory cytokines, and many of the symptoms associated with a classic shock-like syndrome were not present (23). It thus remains unclear what role, if any, is played by proinflammatory cytokines in anthrax pathogenesis.

In the present study, we describe the development of an anthrax vaccine in which adenovirus is used to immunize mice against D4 of PA. Genetic immunization with full-length PA has been shown to provide protective immunity (14, 19, 45); however, concerns have been raised over introducing a potentially active toxin subunit to individuals at risk of exposure to *B. anthracis* (2). Adenovirus was chosen as a vector because of its ability to generate a rapid and robust humoral response to encoded transgenes (46). A number of studies have demonstrated that adenovirus vectors are able to induce rapid immunity to a variety of pathogens (36, 39, 44). We demonstrate that an adenovirus expressing D4 induces protective immunity against LeTx challenge. In addition, we examined the release of inflammatory cytokines from vaccinated mice that either survived or succumbed to toxin challenge in order to further characterize the mechanism by which LeTx kills its host. We present evidence that death from LeTx is not a direct result of inflammatory-cytokine release.

MATERIALS AND METHODS

Cells and culture conditions. 293 cells are human embryonic kidney cells transformed by the adenovirus E1 region (13). C7 cells are 293 cells expressing the adenovirus DNA polymerase and preterminal protein (16). RAW 264.7 cells are a murine macrophage cell line (ATCC TIB-71). All cells were maintained in Dulbecco's modified Eagle's medium (Gibco-BRL) with 10% fetal bovine serum (DMEM-10), 100 U penicillin/ml, and 100 μg streptomycin/ml at 37°C with 5% CO_2.

Codon optimization and vector construction. Optimization of the PA D4 reading frame for expression in mammalian cells was accomplished using a two-step PCR. Overlapping oligonucleotides (Table 1) were first assembled in a reaction mixture consisting of 20 nM (each) oligonucleotide, 0.2 mM deoxynucleotide triphosphates, 1 mM $MgSO_4$, 1.2 U Platinum Pfx Polymerase (Invitrogen), and 5 μl enhancer solution per 50-μl reaction mixture. Two microliters of the assembly reaction mixture was then amplified with the D4for and D4rev primers, which incorporate BamHI and HindIII sites, respectively. The PCR product was confirmed by sequencing and cloned into pUMVC6 (University of Michigan Vector Core) via BamHI and HindIII for incorporation of the interleukin 2 (IL-2) signal peptide. The resulting vector, pUMVC6-D4, was digested with EcoRI and HindIII, and the insert was subcloned into pACCMV2 (University of Michigan Vector Core) to make pACCMV2-D4.

pACCMV2-D4 was linearized with XmnI and recombined onto an adenovirus type 5 backbone lacking the E3 coding region, dl7001 (37), using Cre recombinase (1). The resulting full-length adenovirus chromosome was transfected into C7 cells for rescue of Ad.D4. After plaque purification and amplification, Ad.D4 was purified by double CsCl centrifugation, dialyzed into storage buffer (20 mM Tris, pH 8.0, 25 mM NaCl, 2.5% glycerol), and titered by fluorescent focus assay as described previously (50). Ad.CMV, an isogenic virus containing no transgene, was constructed and purified in the same way.

TABLE 1. Oligonucleotides used for construction of codon-optimized PA domain 4

Name	Sequence
PA F17	5′-TAAAATCAAACTGAATGCCAAGATGAACATCCTGATCCGGGACAAGCGGTTCCACTATGACCGGAATAACATCGCCGTGG-3′
PA F18	5′-GATCAACAGCAGCACCGAAGGCCTGCTGCTGAACATCGACAAAGATATCCGGAAAATCCTGAGCGGCTATATCGTGGAGA-3′
PA F19	5′-GTACGATATGCTGAACATCAGCAGCCTGCGGCAGGATGGCAAGACCTTTATCGATTTCAAGAAATATAACGATAAACTGC-3′
PA F20	5′-GTACGATATGCTGAACATCAGCAGCCTGCGGCAGGATGGCAAGACCTTTATCGATTTCAAGAAATATAACGATAAACTGC-3′
PA R1	5′-CAGTGGATCCTTATTAGCCGATTTCGTAGCCTTTCTTGCTAAAGATCAGGATTTTTTTGATGCCG-3′
PA R2	5′-TGTTTTCCTTGGTCACGGCATACACGTTCACCTTATAATTGGGATTGCTGATATACAGGGGCAGTTTATCGTTATATTTC-3′
PA R3	5′-TGATGTTCAGCATATCGTACCGGTCGTTGATCACCTCTTTCAGGCCCTCGGTATCTTCGATCTCCACGATATAGCCGCTC-3′
PA R4	5′-CTTCGGTGCTGCTGTTGATCACTTCCCGATGGGCCTCTTTCACCACGCTCTCATCGGCGCCCACGGCGATGTTATTCCGG-3′
D4for[a]	5′-ATCGGGATCCTTCCACTATGACCGGAATAA-3′
D4rev[a]	5′-TCAAGCTTTTAGCCGATTTCGTAGCCTT-3′

[a] Underlined sequences represent BamHI (D4for) and HindIII (D4rev) used for cloning.

Western blotting. To confirm the expression of D4 upon infection, 293 cells were infected at a multiplicity of infection of five infectious particles/cell, and cell lysates and supernatants were collected 24 h postinfection. Forty-five micrograms of cell lysate and 30 μl of supernatant were resolved on a 4 to 20% polyacrylamide gel and transferred to nitrocellulose. For PNGase F treatment, samples were incubated with 20,000 U of PNGase F (New England Biolabs) for 1 h at 37°C after being denatured according to the manufacturer's protocol. Western blotting was performed using a rabbit antiserum raised against PA to detect D4, and the adenovirus type 5 IVa2 protein was detected using a goat antiserum as described previously (28).

Vaccination and lethal toxin challenge. Groups of 7- to 8-week-old female Balb/c mice (Jackson Laboratories) were immunized intramuscularly (i.m.) at 0 and 4 weeks with the indicated doses. Virus preparations were diluted in phosphate-buffered saline (PBS), and 50 μl was injected into each quadriceps muscle. As a positive control, one group was injected subcutaneously with 10 μl of AVA (Bioport, Lansing, MI) diluted in 100 μl PBS. Blood was collected from the retro-orbital sinus before the first injection (preimmune) and at 2 and 5 weeks after the first injection. Serum was separated from blood cells and stored at −80°C until analysis. For LeTx challenge, 48 μg of PA and 20 μg of LF (List Biological Laboratories) were injected intravenously via the tail vein, and the mice were closely monitored for 10 days. All animal procedures were approved by the University of Michigan Committee on the Use and Care of Animals.

Serum analysis. Indirect enzyme-linked immunosorbent assays (ELISAs) were used to quantify the anti-PA antibody response in vaccinated mice. Immulon 96-well Maxisorp plates (Nalge Nunc) were coated with 0.1 μg PA/well by incubation at 4°C in PBS overnight. The wells were washed twice with 0.1% Tween 20 in PBS (PBST) and blocked with 5% dry milk in PBST (PBSTM) for 30 min at room temperature. After the wells were washed twice with PBST, serial twofold dilutions of serum in PBSTM were added to the wells and incubated for 90 min at 37°C. The plates were washed three times with PBST, and 100 μl of horseradish peroxidase-conjugated anti-immunoglobulin G (IgG) (Sigma), anti-

IgG1 (Southern Biotechnology), or anti-IgG2a (Immunology Consultants Laboratory) diluted in PBSTM (1:5,000) was added to each well and incubated at room temperature for 1 h. After the wells were washed four times with PBST, 100 μl of horseradish peroxidase substrate (Bio-Rad) was added to each well and developed for 20 min at room temperature. The reaction was stopped by the addition of 100 μl 2% oxalic acid, and the absorbance was read at 415 nm on a SpectraMax 190 (Molecular Devices). The endpoint titer was defined as the highest dilution at which the optical density at 415 nm was at least 0.1 above that of the background wells (wells receiving no serum).

Lethal-toxin-neutralizing titers were determined essentially as described previously (45). Briefly, 24 h before the assay, 3×10^4 RAW 264.7 cells were plated in each well of a 96-well dish. Pooled sera from groups of mice were serially diluted in DMEM-10 and combined with PA to a final concentration of 200 ng PA/ml. The serum-PA mixture was incubated at 37°C for 1 h to allow antibody binding. LF was added to the mixture to a final concentration of 100 ng LF/ml and 100 ng PA/ml. The growth medium was removed from the RAW 264.7 cells, and 100 μl of the PA-LF-serum mixture was added. After incubation at 37°C for 4 h, the PA-LF-serum mixture was removed and 100 μl DMEM-10 and 50 μl XTT reagent (Roche) was added to each well and incubated at 37°C for 16 h. Cell viability was determined by measuring the optical density at 450 nm. Cells receiving no PA or LF were used to determine 100% cell viability. The lethal-toxin-neutralizing titer was defined as the dilution resulting in 50% protection of cells.

Serum cytokines. Two hours after injection of lethal toxin, blood was collected from the retro-orbital sinus. The levels of IL-6 and IL-1β in the serum were measured using a capture ELISA according to the manufacturer's protocol (BD Pharmingen). Concentrations were determined by comparison to a standard curve generated using purified cytokines.

Statistical analysis. Differences in survival between groups of mice were determined using a log rank test. Comparisons of endpoint titers and cytokine levels between groups of mice were made using Student's *t* test. A *P* value of <0.05 was considered significant.

RESULTS

Construction of an adenovirus expressing D4. Analysis of the *B. anthracis* protective-antigen reading frame revealed that many of the codons were not optimal for expression in mammalian cells. In order to ensure high-level expression of D4, an open reading frame in which codon usage was optimized for mammalian cells was constructed by assembling synthetic oligonucleotides (Table 1). The optimized D4 open reading frame was fused to the IL-2 signal peptide to allow secretion of D4 and was placed under the control of a cytomegalovirus (CMV) immediate-early promoter in an adenovirus serotype 5 vector with E1 and E3 deleted (Fig. 1A). To determine if D4 could be stably expressed in and secreted from mammalian cells, 293 cells were infected with Ad.D4 or Ad.CMV. The cell lysates and supernatants were assayed by Western blotting using a polyclonal antibody against PA (Fig. 1B). Five nanograms of recombinant PA was used as a positive control. A band of approximately 20 kDa was detected in both the lysates and supernatants from cells infected with Ad.D4, but not from cells infected with Ad.CMV, indicating that D4 could be expressed and secreted. The presence of D4 in the supernatants was not due to cell lysis, since adenovirus proteins were not detectable in the supernatants (data not shown).

Sequence analysis of D4 revealed that it contained three potential N-linked glycosylation sites. To determine if D4 was being N glycosylated, cell lysates from Ad.D4- infected cells were treated with PNGase F, which cleaves sugars from asparagine residues. As shown in Fig. 1C, the increased mobility of D4 after PNGase F treatment indicated that it was N glycosylated and confirmed that D4 is routed through the secretory pathway, since this modification occurs in the endoplasmic reticulum (18). As expected, the mobility of the adenovirus protein IVa2 was unchanged, since it is not glycosylated.

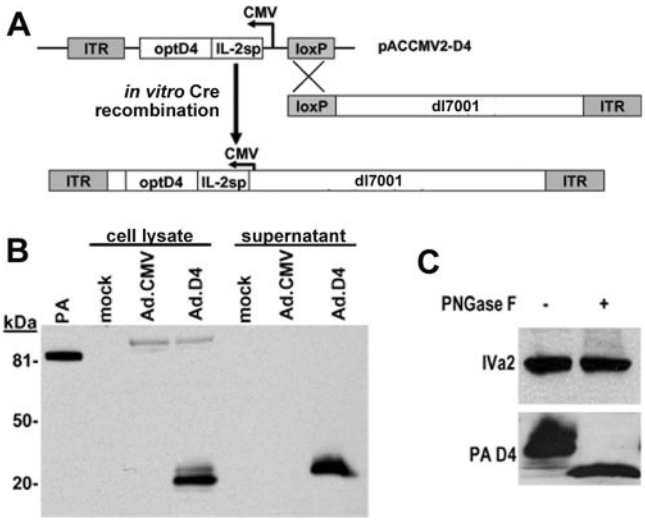

FIG. 1. Construction of Ad.D4 and transgene expression. (A) Schematic showing Ad.D4 construction by Cre recombination between pACCMV2-D4 and the dl7001 backbone. ITR, inverted terminal repeat; CMV, cytomegalovirus immediate-early promoter; optD4, codon-optimized domain 4 of PA; IL-2sp, IL-2 signal peptide. (B) 293 cells were infected at a multiplicity of infection of five infectious particles/cell with either Ad.CMV or Ad.D4 or mock infected. Cell lysates and culture supernatants were analyzed by Western blotting using a polyclonal antibody to PA. (C) Lysates from cells infected with Ad.D4 were untreated (−) or treated (+) with 20,000 U PNGase F, and IVa2 and D4 were detected by Western blotting.

Antibody response. The ability of Ad.D4 to stimulate an antibody response to PA after i.m. administration was determined by injecting groups of five mice with 1×10^7, 1×10^8, or 1×10^9 infectious particles of Ad.D4 at 0 and 4 weeks. As a negative control, one group received 1×10^9 infectious particles of Ad.CMV, the vector encoding no transgene, and as a positive control, one group received 10 μl of AVA subcutaneously. Preimmune serum and serum collected at 2 and 5 weeks were analyzed for the presence of antibodies to PA by indirect ELISA (Fig. 2A). As expected, no antibodies against PA were present in mice before immunization. Two weeks after the first injection, total IgG against PA was detectable in all groups receiving Ad.D4 at levels similar to those receiving AVA. At 5 weeks, total IgG was further increased approximately 10-fold in all groups. Mice receiving Ad.CMV did not have detectable levels of anti-PA antibodies at any time point.

In order to determine the type of immune response being stimulated, the levels of two IgG subtypes were assessed. In mice, IgG1 is indicative of a Th2-type response, whereas IgG2a is predominantly produced during a Th1 response. Two weeks after the first injection, mice receiving the AVA had on average approximately sixfold more IgG1 than mice receiving 1×10^9 infectious particles of Ad.D4 (Fig. 2B), while total IgG levels in the two groups were similar (Fig. 2A). At 5 weeks, IgG1 levels were approximately twofold higher in mice receiving AVA than in mice injected with 1×10^9 infectious particles of Ad.D4. In contrast, mice receiving this dose of Ad.D4 had IgG2a titers of approximately 10^3 2 weeks after injection while AVA-injected mice had no detectable IgG2a at this time point (Fig. 2C). At 5 weeks, IgG2a was detectable in mice receiving

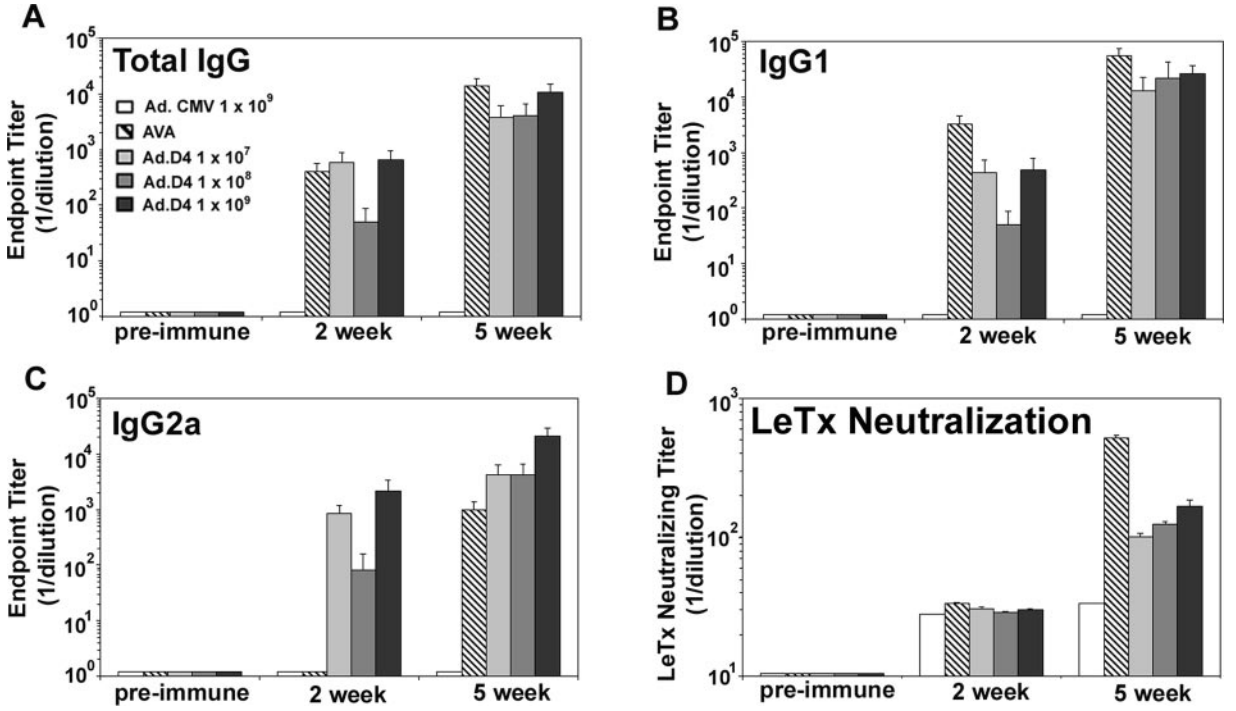

FIG. 2. Antibody responses in vaccinated mice. Anti-PA titers of preimmune, 2-week, and 5-week sera from vaccinated mice. Endpoint titers for total IgG (A), IgG1 (B), and IgG2a (C) are shown as the mean titer for each group, with error bars representing the SEM. (D) LeTx-neutralizing titers of preimmune, 2-week, and 5-week sera from vaccinated mice are expressed as the mean of triplicate experiments, with error bars representing the SEM.

the AVA, although at approximately 25-fold-lower levels than in mice receiving 1×10^9 infectious particles of Ad.D4.

The ability of sera from immunized mice to neutralize toxin in vitro was determined by assessing their ability to protect mouse macrophage cells from LeTx-mediated lysis. At 2 weeks, pooled sera from each group, including the group receiving Ad.CMV, provided protection just above the limit of detection, suggesting that antibodies generated against the adenovirus vector may be able to nonspecifically neutralize LeTx (Fig. 2D). At 5 weeks, neutralizing titers increased significantly in all groups receiving Ad.D4, although to levels approximately three- to fivefold lower than in AVA-injected mice.

Lethal toxin challenge. To determine if administration of two doses of Ad.D4 could provide protection against intoxication, immunized mice were challenged with LeTx. Fourteen days after the final immunization, mice were given four times the 50% lethal dose of LeTx intravenously via the tail vein. All mice receiving Ad.CMV succumbed within 5 days, with a mean time to death of 2.5 ± 0.3 days (mean ± standard error of the mean [SEM]) (Fig. 3A). Of mice vaccinated with 1×10^9 infectious particles of Ad.D4, 67% survived lethal-toxin challenge ($P < 0.001$ versus the Ad.CMV group). The mean time to death of mice vaccinated with Ad.D4 that did not survive (4.3 ± 1.2 days) was approximately 72% longer than that of mice vaccinated with Ad.CMV, although this difference was not statistically significant ($P = 0.067$). All mice in the positive control group (AVA) survived toxin challenge. To verify that survival was correlated with the levels of anti-PA antibodies, prechallenge total IgG titers of vaccinated mice that survived toxin challenge were compared to those of mice that succumbed. On average, mice that survived had approximately 10-fold-higher anti-PA IgG than mice that did not survive (1.2×10^4 versus 1.5×10^3; $P < 0.01$) (Fig. 3B).

Cytokine response to LeTx. To determine whether levels of inflammatory cytokines were different between mice surviving and succumbing to lethal toxin challenge, sera were collected from mice receiving either Ad.D4 ($n = 4$) or Ad.CMV ($n = 4$). The sera were taken 2 hours after toxin administration, since it has been shown that cytokine levels peak at this time point (23). Levels of IL-1β and IL-6 were then measured, because they have been implicated in LeTx-mediated death (15, 35). The IL-1β and IL-6 levels were below the limit of detection in all mice before LeTx injection (Fig. 4). Two hours after LeTx administration, IL-1β levels in survivors and nonsurvivors were not significantly different (139 ± 38 versus 180 ± 22 pg/ml) (Fig. 4A). Furthermore, there was no correlation between the prechallenge anti-PA IgG titer and IL-1β levels in individual mice vaccinated with Ad.D4. Serum levels of IL-6 were significantly higher in mice that survived toxin challenge than in those that did not survive (2,598 ± 249 versus 1,528 ± 47 pg/ml; $P < 0.005$) (Fig. 4B). Mice injected with PA alone, LF alone, or PBS had no detectable IL-1β (data not shown). The IL-6 levels in mice receiving PA and LF alone were similar to levels in mice receiving PBS and approximately fivefold lower than in toxin-injected mice (data not shown), consistent with results seen previously (23).

DISCUSSION

In the context of a bioterrorist incident, it may become necessary to vaccinate large populations against *B. anthracis*. An ideal vaccine would be able to induce rapid protective

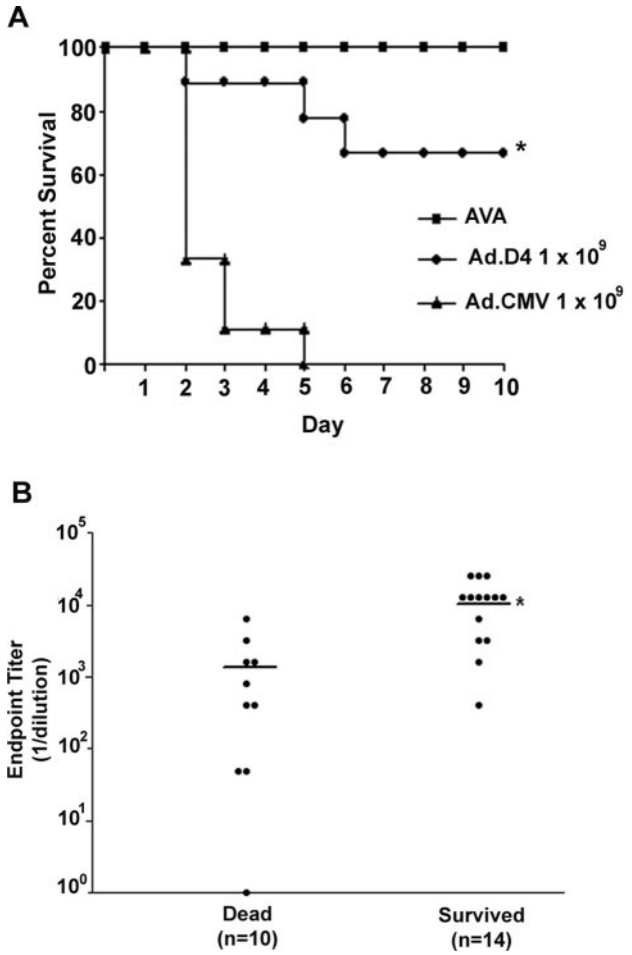

FIG. 3. LeTx challenge. Two weeks after the second immunization, mice were challenged with four times the 50% lethal dose of LeTx intravenously. (A) Kaplan-Meier curve for groups receiving 10 μl AVA, 1×10^9 infectious particles Ad.D4, and 1×10^9 infectious particles Ad.CMV. *, $P < 0.001$ between Ad.D4 and Ad.CMV. (B) Prechallenge total IgG levels for mice surviving and succumbing to toxin challenge. The bars represent the mean titer for each group; *, $P < 0.01$.

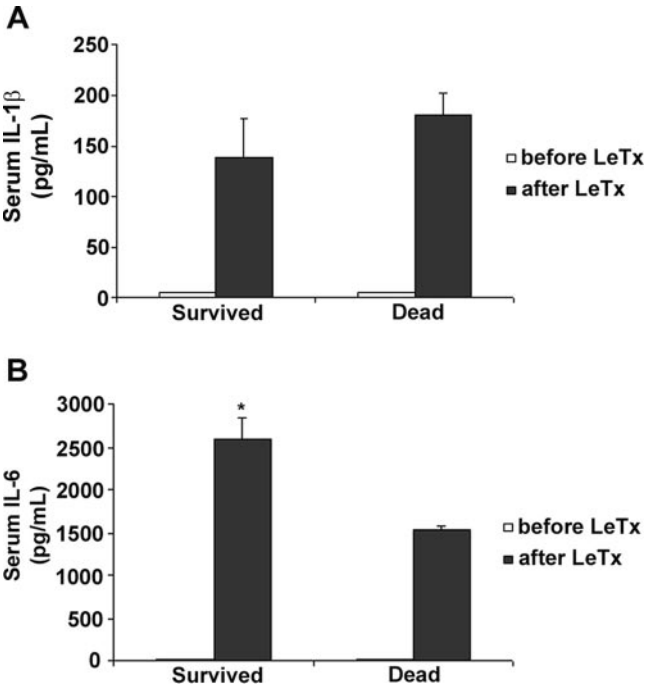

FIG. 4. Cytokine responses to LeTx. Two hours after LeTx administration, sera were collected from mice vaccinated with Ad.CMV ($n = 4$) or Ad.D4 ($n = 4$). Capture ELISAs were used to measure the levels of IL-1β (A) and IL-6 (B) in the sera, and levels from survivors (Survived; $n = 3$) and nonsurvivors (Dead; $n = 5$) were compared. The error bars represent the SEM for each group. *, $P < 0.01$ between IL-6 levels in survivors and nonsurvivors after LeTx challenge.

immunity without causing adverse side effects. The results from this study demonstrate that an adenovirus vector encoding codon-optimized D4 of PA induces an antibody response against PA within 14 days of a single i.m. injection. Furthermore, two doses of 1×10^9 infectious particles administered 4 weeks apart were sufficient to provide significant protection against LeTx challenge. Administration of purified LeTx is an accepted model for the initial evaluation of new anthrax vaccines because it reproduces many symptoms of the disease (24). Vaccination against bacterial challenge would be the next logical test of our vector.

Genetic vaccination using full-length PA expressed from various vectors, including adenovirus, has been shown to be effective for providing protective immunity (14, 19, 45). However, concerns have been raised about administering a potentially active toxin subunit to individuals at risk of being exposed to *B. anthracis* (2). Because it is unknown how toxins made during *B. anthracis* infection will interact with PA produced from genetic vaccination, we chose to use only D4 of PA as a vaccine antigen. D4 is not functional as a toxin subunit, since it lacks the domains necessary for both oligomerization and interaction with the other toxin components, LF and EF (29). The doses of Ad.D4 used in this study (1×10^7 to 1×10^9 infectious particles) are directly scalable for human use, since local administration of 1×10^{12} infectious particles of adenovirus is known to be safe (5). While these doses provided somewhat less protection than AVA (67% versus 100%), it should be noted that the dose of AVA used (10 μl) was not scaled similarly. This amount of AVA, when scaled for use in mice by weight/volume ratio, would be equivalent to approximately 60 times the dose approved for use in humans. In fact, when 1×-scaled doses of the AVA were used to immunize mice, no antibodies against PA were produced (data not shown).

Adenovirus vectors have a number of characteristics that make them attractive for use as vaccine platforms. First, adenovirus has an excellent safety record in humans, as evidenced by its use in many ongoing clinical trials and its previous use as a vaccine in military recruits (http://www.wiley.co.uk/genmed/clinical; 47). Furthermore, adenovirus does not require the addition of reactogenic adjuvants that can produce unwanted side effects. Second, a rapid transgene-specific antibody response is stimulated after a single administration (45, 49). It has also been demonstrated that adenovirus vectors are more efficient at eliciting a humoral response than DNA vaccines and poxvirus vectors (4, 17). In the present study, anti-PA

antibodies induced upon administration of Ad.D4 were predominantly of the IgG2a isotype, whereas the AVA induced mostly IgG1-type antibodies. These results are in agreement with previous work indicating that genetic vaccination with adenovirus results in a robust Th1 response (46). In addition to stimulating a humoral response, vaccination with adenovirus is able to stimulate a strong cytotoxic-T-cell response against the encoded transgene (46). The role of cellular immunity in *B. anthracis* pathogenesis is not clear, although it may be beneficial in combating disease by enabling detection of infected macrophages during the initial stages of infection. Finally, the adenovirus platform can be easily expanded to include new antigens from *B. anthracis* or other pathogens as they are identified.

Concerns have been raised over the utility of adenovirus as a vector due to the presence of preexisting neutralizing antibodies to some serotypes in a significant portion of the population (46). Additionally, readministration of the same vector may be ineffective because of antibodies generated during initial dosing. Here, we have shown that i.m. injection of Ad.D4 4 weeks after a first dose was able to effectively boost antibody titers approximately 10-fold. This suggests either that anti-adenovirus antibodies were not present at sufficient levels to completely neutralize the vector or that anti-adenovirus antibodies were not able to neutralize virus administered intramuscularly. It is therefore possible that additional doses of Ad.D4 would be effective in further boosting anti-PA titers, potentially increasing its ability to elicit protection. While neutralizing antibodies have been shown to reduce the efficiency of adenovirus-mediated gene transfer in some cases (46), a number of strategies have been developed for circumventing this problem. First, it is possible to switch the serotype of the adenovirus vector to a serotype against which immunity is less prevalent in the population being vaccinated (20, 41). Second, the adenovirus capsid can be manipulated so that preexisting antibodies are no longer able to neutralize the vector (25, 26).

Some controversy exists over the role of proinflammatory cytokines in death caused by LeTx. Early evidence suggested that LeTx induces a shock-like syndrome caused by macrophage lysis and subsequent cytokine release. IL-1β was specifically implicated in LeTx-mediated death, since it has been shown that macrophages from susceptible mice release IL-1β in response to LeTx. Furthermore, administration of an IL-1 receptor antagonist with LeTx resulted in complete protection of mice (15). IL-6 has also been implicated in *B. anthracis* pathogenesis, since it is released from primary mouse peritoneal macrophages after infection by spores (31) and mice infected with the fully virulent Ames strain exhibit dramatic increases in serum IL-6 levels (35). However, conflicting reports have shown that LeTx does not induce cytokine release from mouse macrophages (8, 27). Additionally, mice with toxin-resistant macrophages died from LeTx due to hypoxia-induced liver failure in a similar way to those with sensitive macrophages, independent of the release of cytokines (23). In this study, we determined whether levels of inflammatory cytokines correlated with survival after LeTx administration by measuring IL-1β and IL-6 levels in vaccinated mice that either survived or succumbed to toxin challenge. We found that IL-1β levels were similar in surviving and nonsurviving mice. This argues against the notion that the rapid release of IL-1β is responsible for death from anthrax, as has been proposed previously (15). We also showed that IL-6 levels were significantly higher in mice surviving toxin challenge than in those that did not survive, although the biological significance of higher IL-6 levels in survivors versus nonsurvivors is not clear. Interestingly, previous work had shown that serum IL-6 levels were higher in resistant mice than in susceptible mice after spore challenge (35). Taken together, our data are consistent with work indicating that death from LeTx is not a direct result of the release of inflammatory cytokines and the subsequent development of shock (23). This has implications for developing therapies for treating acute anthrax. For example, the model proposing that LeTx causes death due to inflammatory-cytokine release has prompted the development of anticytokine therapies for the treatment of anthrax, similar to those used in the treatment of lipopolysaccharide-induced sepsis (15, 43). Our data suggest that these therapies may be ineffective, since cytokine release occurs regardless of survival.

In summary, the work presented here demonstrates that an adenovirus vector encoding codon-optimized D4 of PA can stimulate protective immunity after two administrations 4 weeks apart. This strategy thus warrants further investigation for use in immunization prior to, or in response to, a bioterrorist event. Additionally, analysis of serum cytokine levels after LeTx challenge suggests that they are not directly responsible for toxin-mediated death. Further investigation of the molecular events leading to LeTx-induced death is thus necessary.

ACKNOWLEDGMENTS

We thank members of the Imperiale and Ferrara laboratories for help with this work and critical review of the manuscript. We thank Vic DiRita and Kathy Spindler for critical review of the manuscript. We also thank the University of Michigan Vector Core for helpful advice and reagents provided under grant 5 P30 CA46592.

This work was supported by R21 AI059231 awarded to M.J.I. from the NIH and the Faculty Research Venture Fund from Frederick G. Novy III and family in honor of Frederick G. Novy. M.J.M was supported by T32 GM07863 and T32 GM08353 from the NIH.

REFERENCES

1. **Aoki, K., C. Barker, X. Danthinne, M. J. Imperiale, and G. J. Nabel.** 1999. Efficient generation of recombinant adenoviral vectors by Cre-lox recombination in vitro. Mol. Med. **5:**224–231.
2. **Aulinger, B. A., M. H. Roehrl, J. J. Mekalanos, R. J. Collier, and J. Y. Wang.** 2005. Combining anthrax vaccine and therapy: a dominant-negative inhibitor of anthrax toxin is also a potent and safe immunogen for vaccines. Infect. Immun. **73:**3408–3414.
3. **Brossier, F., M. Weber-Levy, M. Mock, and J. C. Sirard.** 2000. Role of toxin functional domains in anthrax pathogenesis. Infect. Immun. **68:**1781–1786.
4. **Casimiro, D. R., L. Chen, T. M. Fu, R. K. Evans, M. J. Caulfield, M. E. Davies, A. Tang, M. Chen, L. Huang, V. Harris, D. C. Freed, K. A. Wilson, S. Dubey, D. M. Zhu, D. Nawrocki, H. Mach, R. Troutman, L. Isopi, D. Williams, W. Hurni, Z. Xu, J. G. Smith, S. Wang, X. Liu, L. Guan, R. Long, W. Trigona, G. J. Heidecker, H. C. Perry, N. Persaud, T. J. Toner, Q. Su, X. Liang, R. Youil, M. Chastain, A. J. Bett, D. B. Volkin, E. A. Emini, and J. W. Shiver.** 2003. Comparative immunogenicity in rhesus monkeys of DNA plasmid, recombinant vaccinia virus, and replication-defective adenovirus vectors expressing a human immunodeficiency virus type 1 *gag* gene. J. Virol. **77:**6305–6313.
5. **Crystal, R. G., B. G. Harvey, J. P. Wisnivesky, K. A. O'Donoghue, K. W. Chu, J. Maroni, J. C. Muscat, A. L. Pippo, C. E. Wright, R. J. Kaner, P. L. Leopold, P. D. Kessler, H. S. Rasmussen, T. K. Rosengart, and C. Hollmann.** 2002. Analysis of risk factors for local delivery of low- and intermediate-dose adenovirus gene transfer vectors to individuals with a spectrum of comorbid conditions. Hum. Gene Ther. **13:**65–100.
6. **Dixon, T. C., M. Meselson, J. Guillemin, and P. C. Hanna.** 1999. Anthrax. N. Engl. J. Med. **341:**815–826.
7. **Duesbery, N. S., C. P. Webb, S. H. Leppla, V. M. Gordon, K. R. Klimpel,**

T. D. Copeland, N. G. Ahn, M. K. Oskarsson, K. Fukasawa, K. D. Paull, and G. F. Vande Woude. 1998. Proteolytic inactivation of MAP-kinase-kinase by anthrax lethal factor. Science **280:**734–737.
8. **Erwin, J. L., L. M. DaSilva, S. Bavari, S. F. Little, A. M. Friedlander, and T. C. Chanh.** 2001. Macrophage-derived cell lines do not express proinflammatory cytokines after exposure to *Bacillus anthracis* lethal toxin. Infect. Immun. **69:**1175–1177.
9. **Fellows, P. F., M. K. Linscott, B. E. Ivins, M. L. Pitt, C. A. Rossi, P. H. Gibbs, and A. M. Friedlander.** 2001. Efficacy of a human anthrax vaccine in guinea pigs, rabbits, and rhesus macaques against challenge by *Bacillus anthracis* isolates of diverse geographical origin. Vaccine **19:**3241–3247.
10. **Flick-Smith, H. C., N. J. Walker, P. Gibson, H. Bullifent, S. Hayward, J. Miller, R. W. Titball, and E. D. Williamson.** 2002. A recombinant carboxy-terminal domain of the protective antigen of *Bacillus anthracis* protects mice against anthrax infection. Infect. Immun. **70:**1653–1656.
11. **Friedlander, A. M., R. Bhatnagar, S. H. Leppla, L. Johnson, and Y. Singh.** 1993. Characterization of macrophage sensitivity and resistance to anthrax lethal toxin. Infect. Immun. **61:**245–252.
12. **Geier, D. A., and M. R. Geier.** 2002. Anthrax vaccination and joint related adverse reactions in light of biological warfare scenarios. Clin. Exp. Rheumatol. **20:**217–220.
13. **Graham, F. L., J. Smiley, W. C. Russell, and R. Nairn.** 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. **36:**59–74.
14. **Gu, M. L., S. H. Leppla, and D. M. Klinman.** 1999. Protection against anthrax toxin by vaccination with a DNA plasmid encoding anthrax protective antigen. Vaccine **17:**340–344.
15. **Hanna, P. C., D. Acosta, and R. J. Collier.** 1993. On the role of macrophages in anthrax. Proc. Natl. Acad. Sci. USA **90:**10198–10201.
16. **Hartigan-O'Connor, D., A. Amalfitano, and J. S. Chamberlain.** 1999. Improved production of gutted adenovirus in cells expressing adenovirus preterminal protein and DNA polymerase. J. Virol. **73:**7835–7841.
17. **He, Z., A. P. Wlazlo, D. W. Kowalczyk, J. Cheng, Z. Q. Xiang, W. Giles-Davis, and H. C. Ertl.** 2000. Viral recombinant vaccines to the E6 and E7 antigens of HPV-16. Virology **270:**146–161.
18. **Helenius, A., and M. Aebi.** 2001. Intracellular functions of N-linked glycans. Science **291:**2364–2369.
19. **Hermanson, G., V. Whitlow, S. Parker, K. Tonsky, D. Rusalov, M. Ferrari, P. Lalor, M. Komai, R. Mere, M. Bell, K. Brenneman, A. Mateczun, T. Evans, D. Kaslow, D. Galloway, and P. Hobart.** 2004. A cationic lipid-formulated plasmid DNA vaccine confers sustained antibody-mediated protection against aerosolized anthrax spores. Proc. Natl. Acad. Sci. USA **101:**13601–13606.
20. **Holterman, L., R. Vogels, R. van der Vlugt, M. Sieuwerts, J. Grimbergen, J. Kaspers, E. Geelen, E. van der Helm, A. Lemckert, G. Gillissen, S. Verhaagh, J. Custers, D. Zuijdgeest, B. Berkhout, M. Bakker, P. Quax, J. Goudsmit, and M. Havenga.** 2004. Novel replication-incompetent vector derived from adenovirus type 11 (Ad11) for vaccination and gene therapy: low seroprevalence and non-cross-reactivity with Ad5. J. Virol. **78:**13207–13215.
21. **Ivins, B. E., and S. L. Welkos.** 1988. Recent advances in the development of an improved, human anthrax vaccine. Eur. J. Epidemiol. **4:**12–19.
22. **Larkin, M.** 2002. Anthrax vaccine is safe and effective-but needs improvement, says IOM. Lancet **359:**951.
23. **Moayeri, M., D. Haines, H. A. Young, and S. H. Leppla.** 2003. *Bacillus anthracis* lethal toxin induces TNF-alpha-independent hypoxia-mediated toxicity in mice. J. Clin. Investig. **112:**670–682.
24. **Moayeri, M., and S. H. Leppla.** 2004. The roles of anthrax toxin in pathogenesis. Curr. Opin. Microbiol. **7:**19–24.
25. **O'Riordan, C. R., A. Lachapelle, C. Delgado, V. Parkes, S. C. Wadsworth, A. E. Smith, and G. E. Francis.** 1999. PEGylation of adenovirus with retention of infectivity and protection from neutralizing antibody in vitro and in vivo. Hum. Gene Ther. **10:**1349–1358.
26. **Ostapchuk, P., and P. Hearing.** 2001. Pseudopackaging of adenovirus type 5 genomes into capsids containing the hexon proteins of adenovirus serotypes B, D, or E. J. Virol. **75:**45–51.
27. **Pellizzari, R., C. Guidi-Rontani, G. Vitale, M. Mock, and C. Montecucco.** 2000. Lethal factor of *Bacillus anthracis* cleaves the N-terminus of MAPKKs: analysis of the intracellular consequences in macrophages. Int. J. Med. Microbiol. **290:**421–427.
28. **Perez-Romero, P., R. E. Tyler, J. R. Abend, M. Dus, and M. J. Imperiale.** 2005. Analysis of the interaction of the adenovirus L1 52/55-kilodalton and IVa2 proteins with the packaging sequence in vivo and in vitro. J. Virol. **79:**2366–2374.
29. **Petosa, C., R. J. Collier, K. R. Klimpel, S. H. Leppla, and R. C. Liddington.** 1997. Crystal structure of the anthrax toxin protective antigen. Nature **385:**833–838.
30. **Pezard, C., P. Berche, and M. Mock.** 1991. Contribution of individual toxin components to virulence of *Bacillus anthracis*. Infect. Immun. **59:**3472–3477.
31. **Pickering, A. K., M. Osorio, G. M. Lee, V. K. Grippe, M. Bray, and T. J. Merkel.** 2004. Cytokine response to infection with *Bacillus anthracis* spores. Infect. Immun. **72:**6382–6389.
32. **Pitt, M. L., S. F. Little, B. E. Ivins, P. Fellows, J. Barth, J. Hewetson, P. Gibbs, M. Dertzbaugh, and A. M. Friedlander.** 2001. In vitro correlate of immunity in a rabbit model of inhalational anthrax. Vaccine **19:**4768–4773.
33. **Pittman, P. R., P. H. Gibbs, T. L. Cannon, and A. M. Friedlander.** 2001. Anthrax vaccine: short-term safety experience in humans. Vaccine **20:**972–978.
34. **Pittman, P. R., G. Kim-Ahn, D. Y. Pifat, K. Coonan, P. Gibbs, S. Little, J. G. Pace-Templeton, R. Myers, G. W. Parker, and A. M. Friedlander.** 2002. Anthrax vaccine: immunogenicity and safety of a dose-reduction, route-change comparison study in humans. Vaccine **20:**1412–1420.
35. **Popov, S. G., T. G. Popova, E. Grene, F. Klotz, J. Cardwell, C. Bradburne, Y. Jama, M. Maland, J. Wells, A. Nalca, T. Voss, C. Bailey, and K. Alibek.** 2004. Systemic cytokine response in murine anthrax. Cell Microbiol. **6:**225–233.
36. **Prevec, L., J. B. Campbell, B. S. Christie, L. Belbeck, and F. L. Graham.** 1990. A recombinant human adenovirus vaccine against rabies. J. Infect. Dis. **161:**27–30.
37. **Ranheim, T. S., J. Shisler, T. M. Horton, L. J. Wold, L. R. Gooding, and W. S. Wold.** 1993. Characterization of mutants within the gene for the adenovirus E3 14.7-kilodalton protein which prevents cytolysis by tumor necrosis factor. J. Virol. **67:**2159–2167.
38. **Reuveny, S., M. D. White, Y. Y. Adar, Y. Kafri, Z. Altboum, Y. Gozes, D. Kobiler, A. Shafferman, and B. Velan.** 2001. Search for correlates of protective immunity conferred by anthrax vaccine. Infect. Immun. **69:**2888–2893.
39. **Reyes-Sandoval, A., J. C. Fitzgerald, R. Grant, S. Roy, Z. Q. Xiang, Y. Li, G. P. Gao, J. M. Wilson, and H. C. Ertl.** 2004. Human immunodeficiency virus type 1-specific immune responses in primates upon sequential immunization with adenoviral vaccine carriers of human and simian serotypes. J. Virol. **78:**7392–7399.
40. **Santelli, E., L. A. Bankston, S. H. Leppla, and R. C. Liddington.** 2004. Crystal structure of a complex between anthrax toxin and its host cell receptor. Nature **430:**905–908.
41. **Seshidhar Reddy, P., S. Ganesh, M. P. Limbach, T. Brann, A. Pinkstaff, M. Kaloss, M. Kaleko, and S. Connelly.** 2003. Development of adenovirus serotype 35 as a gene transfer vector. Virology **311:**384–393.
42. **Sever, J. L., A. I. Brenner, A. D. Gale, J. M. Lyle, L. H. Moulton, B. J. Ward, and D. J. West.** 2004. Safety of anthrax vaccine: an expanded review and evaluation of adverse events reported to the Vaccine Adverse Event Reporting System (VAERS). Pharmacoepidemiol. Drug. Saf. **13:**825–840.
43. **Shin, S., G. H. Hur, Y. B. Kim, G. B. Yeon, K. J. Park, Y. M. Park, and W. S. Lee.** 2000. Dehydroepiandrosterone and melatonin prevent *Bacillus anthracis* lethal toxin-induced TNF production in macrophages. Cell Biol. Toxicol. **16:**165–174.
44. **Sullivan, N. J., T. W. Geisbert, J. B. Geisbert, L. Xu, Z. Y. Yang, M. Roederer, R. A. Koup, P. B. Jahrling, and G. J. Nabel.** 2003. Accelerated vaccination for Ebola virus haemorrhagic fever in non-human primates. Nature **424:**681–684.
45. **Tan, Y., N. R. Hackett, J. L. Boyer, and R. G. Crystal.** 2003. Protective immunity evoked against anthrax lethal toxin after a single intramuscular administration of an adenovirus-based vaccine encoding humanized protective antigen. Hum. Gene Ther. **14:**1673–1682.
46. **Tatsis, N., and H. C. Ertl.** 2004. Adenoviruses as vaccine vectors. Mol. Ther. **10:**616–629.
47. **Top, F. H., Jr., B. A. Dudding, P. K. Russell, and E. L. Buescher.** 1971. Control of respiratory disease in recruits with types 4 and 7 adenovirus vaccines. Am. J. Epidemiol. **94:**142–146.
48. **Williamson, E. D., I. Hodgson, N. J. Walker, A. W. Topping, M. G. Duchars, J. M. Mott, J. Estep, C. Lebutt, H. C. Flick-Smith, H. E. Jones, H. Li, and C. P. Quinn.** 2005. Immunogenicity of recombinant protective antigen and efficacy against aerosol challenge with anthrax. Infect. Immun. **73:**5978–5987.
49. **Xiang, Z. Q., Y. Yang, J. M. Wilson, and H. C. Ertl.** 1996. A replication-defective human adenovirus recombinant serves as a highly efficacious vaccine carrier. Virology **219:**220–227.
50. **Zhang, W., and M. J. Imperiale.** 2003. Requirement of the adenovirus IVa2 protein for virus assembly. J. Virol. **77:**3586–3594.

Editor: J. T. Barbieri